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PRINCIPAL INVESTIGATOR: Jim Klostergaard, Ph.D.

CONTRACTING ORGANIZATION: University of Texas  
M.D. Anderson Cancer Center  
Houston, Texas 77030

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<b>13. ABSTRACT (Maximum 200 Words)</b> <p>We have observed that certain sphingolipids (e.g., dimethyl-sphingosine: DMSP) induce apoptosis <i>in vitro</i> in tumor cells despite the over-expression of HER-2/neu and other resistance mechanisms relevant to breast cancer. In these studies, we translated formulation and toxicity studies of liposomal-DMSP (L-DMSP) to proof-of-principle efficacy studies in the nude mouse/human HER-2/neu over-expressing MDA-MD-361 breast adenocarcinoma model. In the first tier efficacy experiments, mice were treated with a multiple-dose MTD regimen of L-DMSP administered i.v. beginning either one-week after tumor implantation or when tumors grew to 4-5 mm diameter. Early treatment caused a delay in or reduced subsequent tumor growth, but was rarely curative; the tumor growth curve was suggestive of stasis. Treatment initiated at the later timepoint was also efficacious, but less so; the response appeared to be primarily very brief stasis, with slight growth occurring through treatment, followed by a slower growth rate than for controls. In the second tier efficacy experiments using the later timepoint only, the effect of PEG in the liposomal formulation was evaluated, and the i.p. and i.v. routes of administration were compared. The PEG formulation appeared superior to the non-PEG, and the i.p. route did not prove efficacious.</p>				
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## Introduction

Over-expression of HER-2/neu has been linked to poorer prognosis and reduced survival in cancer patients, including breast cancer patients. The basis for this association is likely multifactorial and includes therapeutic resistance, such as resistance to Taxol (paclitaxel), widely used in many chemotherapeutic regimens for this disease. We have recently observed that certain sphingolipids (e.g., dimethyl-sphingosine), either as free lipids or as constituents of liposomes, induce apoptosis *in vitro* in tumor cells despite the over-expression of HER-2/neu, P-gp-170 and other resistance mechanisms relevant to breast cancer.

The purpose of these studies was ultimately to translate the formulation and toxicity studies of liposomal-dimethyl-sphingosine (L-DMSP) conducted in the initial phase of this grant to proof-of-principle efficacy studies in nude mouse/human HER-2/neu over-expressing breast adenocarcinoma models. Among the numerous choices of models, the one that in our hands best fit the multiple criteria including orthotopic growth, high tumorigenicity and stable, high-level expression of HER-2/neu was the human breast adenocarcinoma, MDA-MB-361. Investigations leading to and pertinent to the efficacy studies were the main focus in the un-funded extension year. We present herein evaluation of the anti-tumor efficacy of different formulations of L-DMSP in the MDA-MB-361 model.

Progress in the efficacy studies was reasonable and these studies are continuing with the intent to develop additional data for future grant applications.

## Body

### Task 4

Initial formulations of long-circulating (PEG-containing) liposomes (PEG-L-DMSP) have been prepared by the lipid film hydration and extrusion methods. The composition was DMSP/DPPC/DSPE, 1:2:2:0.4. No particular difficulties in preparing this formulation were encountered.

We have recently gravitated toward the extrusion technique for liposome preparation in preference to the sonication method we have previously used for many of our studies. Extrusion through progressively smaller-diameter membrane pores 1) readily lends itself to scale-up, 2) avoids the high transient temperature gradients associated with sonication that otherwise might result in lipid oxidation or other damage, and 3) results in a final liposome preparation with well-defined average diameters. We have prepared both the PEG-L-DMSP formulations in this manner, as well as the conventional DMSP liposomes.

### Tasks 5 and 6

In the first year, studies in nude mice indicated that a multiple-dose MTD for DMSP (as L-DMSP) of 4.0 mg was a more accurate figure than the 0.5-1.5 mg previously suggested by the literature and by our own early preliminary studies. These studies were completed and confirmed a value of 4.0-4.5 mg per injection for the standard (non-stealth/non-PEG) formulation (DMSP/DPPC/DSPE, 1:2:2) in a multiple-dose regimen. These liposomal formulations were prepared by the sonication as opposed to extrusion technique. We have unexpectedly observed an apparent linkage between a higher incidence of drug-toxicity in more recent experiments in tumor-bearing mice when we have employed the extruded rather than sonicated liposome formulations. The cause of this apparent association is unclear. One speculation is that the extruded formulations

undergo less damage during preparation than with the sonication technique, making more DMSP bio-available, therefore resulting in greater toxicity.

#### Task 7

Task 7, using long-circulating (PEG-containing) liposomes were narrowed to nude mouse studies, foregoing studies in BALB/c mice.

#### Task 8

SUV liposome formulations of DMSP (L-DMSP) were prepared by lipid film hydration and either extensive sonication or repeated extrusion techniques. The mole composition was DMSP/DPPC/DSPC, 1:2:2.

MDA-MB-361 human HER-2/neu-over-expressing breast adenocarcinoma cells were obtained from ATCC and cultured in CO<sub>2</sub>-free Liebowitz L-15 medium; these specific culture conditions were required to maintain the original cell morphology and tumorigenicity.  $4-6 \times 10^6$  cells were implanted in the mammary fat pad of 6-9 week old female nude mice. In the first experiments, mice were treated with a multiple-dose MTD regimen of L-DMSP (4.5 mg DMSP per injection, i.v.) beginning either one-week later or when tumors grew to 4-5 mm diameter. Tumor growth was monitored by caliper measurements; the product of these diameters was taken to reflect tumor areas (Fig. 1 and 2).

Early treatment (one week after tumor implantation) with a multiple-dose (five injections over about two weeks) regimen of L-DMSP (4.5 mg DMSP per injection; 20 mole percent of an SUV formulation), caused a delay in or reduced subsequent tumor growth, but was apparently curative in only one of eight mice (Fig. 1 and 2). The tumor growth curve was suggestive of stasis, with anti-tumor effects evident for as long as ~30 days after cessation of treatment; the caveat in this experiment was two toxic deaths that occurred in this group within a week after the last injection. When the five injections were administered over a slightly longer timeframe (16 vs. 14 days) or to mice that were ~4 weeks older, no deaths occurred, and a slower rate of tumor growth than for the controls was still observed.

When administration was initiated at the later time point (tumor diameters, 4-5 mm), treatment with L-DMSP was also efficacious, but less so than with early treatment. The effects of late treatment with L-DMSP (Fig. 1) appeared to be primarily very brief stasis, with slight growth occurring through treatment, followed by a slower growth rate than for controls.

#### Task 9

This Task was undertaken once results from Task 8 were available, allowing comparisons of the anti-tumor efficacy of long-circulating, PEG-SUVs to those of the non-targeted, conventional SUVs. As before,  $4-6 \times 10^6$  cells were implanted in the mammary fat pad of 6-9 week old female nude mice. Mice were treated in four separate groups once their tumors grew to 4-5 mm average diameter for the entire cohort. The groups included 1) treatment with a multiple-dose regimen of L-DMSP administered i.v.; 2) treatment with a multiple-dose regimen of L-DMSP administered i.p.; 3) treatment with a multiple-dose regimen of PEG-L-DMSP i.v.; and 4) treatment with a multiple-dose regimen of PEG-L-DMSP i.p.

We began these regimens with a dose of 4.5 mg DMSP, based on considerable experience that indicated that this dose is usually well-tolerated. However, numerous mice perished in the 24-48 hr after administration of this first dose, so we adjusted the subsequent doses to 2.25 mg per dose. The realistic MTD in these tumor-bearing mice is most likely between these levels. This is important,



since we have observed steep dose-response effects with DMSP in *in vitro* evaluation against numerous tumor cell lines.

For these studies, the responses of individual mice were tracked. Tumor volumes, calculated as  $l \times w \times w/2$ , were recorded; the fold-increase in tumor volume from the initiation of treatment until the termination of the study 4-5 weeks later was then calculated as a ratio. The ratios for each mouse in a group were used to calculate mean  $\pm$  SEM, and to generate p values to discern significant differences.

Since occasional and unexplained "spontaneous" regressions were observed in the control group, these data and data for any mice in any of the treatment groups with regressing tumors, whether occurring spontaneously or due to treatment, were also censored. While rigorous, this approach to data evaluation carries the risk that bona fide treatment responses, in fact the most striking responses, will not be captured. This unresolved issue is the major drawback to this tumor model.

The results indicated that for controls, the fold-increase in tumor volume was  $4.83 \pm 1.21$ . For the L-DMSP administered i.v. group, the ratio was  $3.48 \pm 1.40$  ( $p = 0.49$  vs. control); for the L-DMSP administered i.p. group, the ratio was  $4.49 \pm 1.12$  ( $p = 0.87$  vs. control); for the PEG-L-DMSP administered i.v. group, the ratio was  $1.78 \pm 0.21$  ( $p = 0.17$  vs. control); and for the PEG-L-DMSP administered i.p. group, the ratio was  $4.80 \pm 0.72$  ( $p = 0.98$  vs. control). The p value for comparison between the PEG-L-DMSP groups administered i.v. vs. i.p. was 0.55.

The trend indicated that 1) PEG-L-DMSP administered i.v. afforded the best response, missing significance in part due to the small numbers of mice (partly a result of the early toxic deaths), 2) comparing i.p. vs. i.v. administration, the former was ineffective with this schedule and dose, and 3) the response with the 2.25 mg DMSP per injection dose level was not as compelling as with the 4.5 used in the previous experiments. There appears to be a very narrow window between drug toxicity and anti-tumor efficacy in this model and with this formulation and dose/schedule.

It must be pointed out that the MDA-MB-361 model is quite rigorous in terms of its drug resistance. For example, it was highly resistant to a multiple-dose regimen of Taxol administered near its MTD, at up to 10 mg/kg i.p. on a qd7 X 3 schedule. In this experiment, the fold-increase in tumor volumes for controls was  $5.89 \pm 0.43$ ; for the 5mg/kg Taxol group, it was  $6.06 \pm 0.25$  ( $p < 0.74$  vs control); for the 10 mg/kg Taxol group, it was  $4.29 \pm 0.61$  ( $p = 0.10$  vs control). The p value for the comparison between the two Taxol groups was 0.035. Thus, defining even modest anti-tumor activity for DMSP formulations in this HER-2/neu over-expressing model has merit in our view. To date, only a novel pro-drug copolymer formulation of paclitaxel covalently linked to poly-L-glutamic acid has shown marked anti-tumor activity in this model ( $p = 0.0006$  vs control; in preparation).

The next experiment, which will be launched with tumor implantation next week, will focus solely on i.v. arms, to the exclusion of i.p. arms, to more rigorously compare the L-DMSP vs. PEG-L-DMSP formulations; this is intended to determine whether the longer circulation time and resultant increased tumor accumulation associated with PEG-liposomes will result in greater anti-tumor activity with DMSP. 10 mice per group are planned, to increase the opportunity to demonstrate statistical significance. We are considering an intermediate dose level between 2.25 mg and 4.5 mg, to balance the toxicity and efficacy concerns cited above. In addition, we will include

an arm examining an even higher dose of Taxol, 15 mg/kg, very close to or possibly exceeding its MTD, to better establish the resistance profile of this model.

#### Tasks 10 and 11

We placed these studies in a lower priority than the nude mouse studies, and did not undertake them in light of the emphasis on the mouse models.

#### Tasks 12 and 13

These studies were certainly planned, but took a lower priority than establishing the HER-2/neu tumor model for evaluation of anti-tumor efficacy. They were ultimately not conducted, due to the complications associated with the former model.

#### Task 14

The most critical experiments to repeat were those supporting the proof-of-principle, anti-tumor efficacy studies in the MDA-MB-361 model.

### **Key Research Accomplishments**

Re-established human MDA-MB-361 HER-2/neu-over-expressing orthotopic human breast adenocarcinoma xenograft model in female nude mice

Identified positive, durable anti-tumor efficacy of a multiple-dose MTD regimen of L-DMSP (conventional SUVs) in the 361 model, with both early (one week post-tumor implantation) and late (tumor diameters, 4-5 mm) treatments

Identified anti-tumor efficacy of a multiple-dose MTD regimen of PEG-L-DMSP in the 361 model with late (tumor diameters, 4-5 mm) treatment, and the superiority of i.v. vs. i.p. administration

### **Reportable Outcomes**

Two abstracts (Era of Hope Meeting, September, 2002, Orlando, FL, "Liposomal-dimethylsphingosine and paclitaxel copolymer are active against HER-2/neu-overexpressing human breast adenocarcinoma orthotopic xenograft model"; EORTC/AACR/NCI Meeting, November, 2002, Frankfurt, Germany, "Evaluation *in vivo* of new agents for drug-resistant ovarian and breast carcinomas") were accepted and poster presentations have been given.

Two manuscripts are in preparation: the first describing the effects of DMSP *in vitro* on HER-2/neu expression levels and on the Akt/PI-3K pathway in human breast adenocarcinomas, and the second on the toxicity and efficacy studies in the MDA-MB-361 model, the later pending the completion of the next L-DMSP and PEG-L-DMSP efficacy experiments.

We have submitted a grant application to the 2003 DOD Breast Cancer Research Program intended to extend these results.

### **Conclusions**

We conclude that the sphingolipid, DMSP, administered as a liposomal (SUV) formulation, has anti-tumor efficacy against the HER-2/neu over-expressing MDA-MB-361 human breast adenocarcinoma orthotopic nude mouse xenograft model, evident with either lower or higher tumor burden. Inclusion of PEG in the liposomal formulation enhanced the efficacy, and the i.v. route of administration was superior to the i.p.

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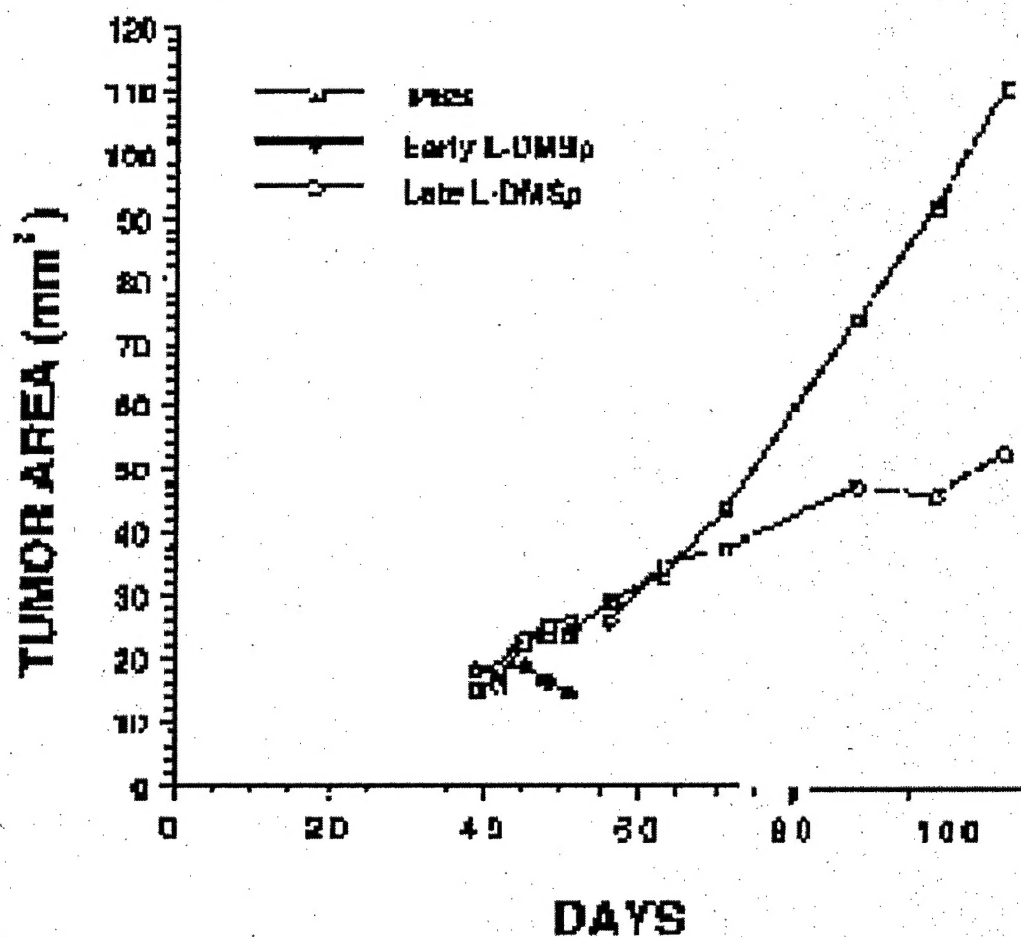
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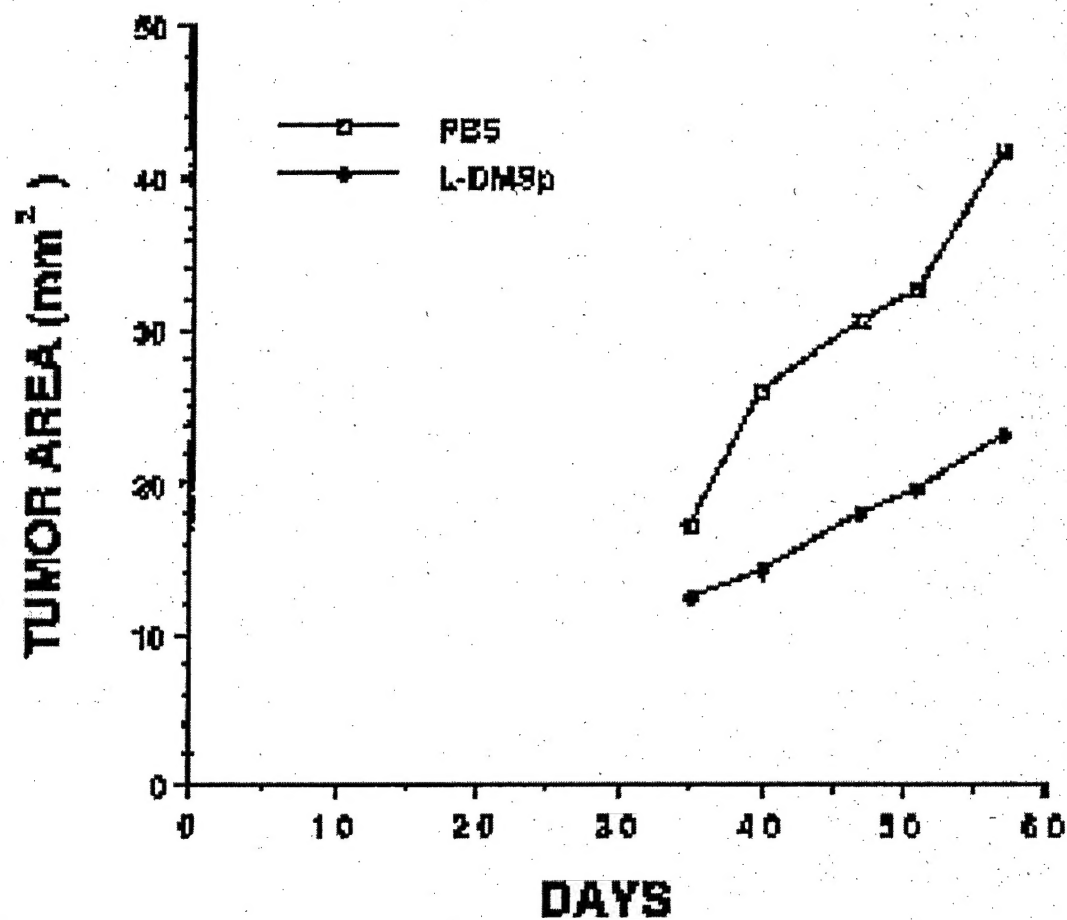
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**Figure 1**

Responses of 361 model to L-DMSP  
L-DMSP was composed of DMSP/DPPC/DSPC. Formulation was administered i.v. on Days 7, 10, 14, 18 and 21 (early treatment), or on Days 38, 42, 45, 48 and 51 (late treatment). Two deaths from drug toxicity occurred in the early treatment group on Days 23 and 28.



**Figure 2**

Responses of 361 model to L-DMSP  
 L-DMSP was composed of DMSP/DPPC/DSPC. Formulation was administered i.v. on Days 7, 12, 16, 19 and 23. No deaths from drug toxicity occurred.